

CONTRIBUTION TO OUR KNOWLEDGE OF THE CHEMISTRY OF BLOOD.

NO 1. GLOBIN SULPHATE AND GLOBIN FROM OX-BLOOD.

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General considerations.—Oxyhæmoglobin from ox-blood has received very little attention as regards its chemical composition, and although, as is well known, this is due to the fact that the substance is not readily crystallisable, nevertheless it is not a little surprising in view of the fact that a solution of ox-blood is universally accepted as a standard solution of hæmoglobin. The fact that hæmoglobins behave differently in their properties, especially as regards their power of crystallising, is a clear indication of a difference in chemical constitution, and hence in the case of ox-blood hæmoglobin we might well expect this difference to be considerable. That such is the case is demonstrated by the results put forward in this communication. The researches of Küster* and others have almost conclusively shown that these differences are not due to any variation in the composition of the prosthetic group, and moreover since this portion of the hæmoglobin molecule constitutes only five per cent. of the whole, it cannot account for the marked differences in hæmoglobins from various sources. The difference must, therefore, certainly lie with the albuminous moiety, and this is borne out by experiment.

I have been able to show that the globin of ox-blood is more basic than that from the hæmoglobin of the horse, which has served as the material of most previous investigations. This basicity is evidenced by the power of the histone to combine with nearly nine per cent. of sulphuric acid.

* H. S. Zeit. Physiol. Chem. Bd. 40, S. 391, 1904.

EXPERIMENTAL.—*The preparation of globin from ox-blood by Lawrow's method **

The Lawrow method is briefly as follows. An aqueous solution of hæmoglobin is poured, with constant agitation, into a large excess of a mixture of five parts of alcohol, two parts ether, and one water, the mixture having been rendered of such an acidity that 100cc. contain 0.025 grams H_2SO_4 . It is necessary to add the hæmoglobin solution with care, otherwise the precipitate is likely to be contaminated with undecomposed hæmoglobin which is difficult to remove by washing. This difficulty can, however, be readily overcome by using a much larger excess of sulphuric acid than was employed by Lawrow.

In the application of Lawrow's method to the precipitation of ox-blood globin, it is necessary, on account of the greater basicity of the globin, to employ more sulphuric acid, as will be seen from the following experiment.

EXPERIMENT 1.

Alcohol.....	1000cc.
Ether.....	400cc.
Water.....	120cc.
H_2SO_4 N/10.....	80cc.

To this mixture were added 50cc. of blood-solution containing the hæmoglobin from exactly 25cc. of defibrinated ox-blood. The solution was thoroughly shaken during the addition of the hæmoglobin in a stoppered bottle, and the flocculent white precipitate allowed to settle. Of the hæmoglobin solution, 50cc. more were now cautiously added, with constant shaking; the precipitate which formed was of deep red colour, and moreover the solution did not darken as before, indicating that the further quantity of hæmoglobin had not been decomposed. A portion of the supernatant fluid was tested, and found almost neutral to methyl orange, and neutral to dimethylamidoazobenzol, but acid to phenolphthalein. Moreover, on addition of a large excess of sulphuric acid, the precipitated hæmoglobin was immediately split, the hæmatin dissolving and darkening the solution, and the red colour of the precipitate giving place to the white of the

* H. S. Zeit. Physiol. Chem. Bd. 26, S. 343, 1898.

globin. There is another interest attached to this experiment. As was pointed out, the supernatant fluid was acid towards phenolphthalein, although neutral to methyl orange.

Of the supernatant fluid, 250cc. required 1.0cc. N/10 Na_2CO_3 to render the solution distinctly alkaline to methyl orange. Of the same solution, 250cc required 3.3cc. N/10 NaOH to produce the first pink colour with phenolphthalein. It is clear, therefore, that organic acids are produced in the solution during the decomposition of hæmoglobin with sulphuric acid. This fact has been pointed out before by other observers.

Hæmatin was completely removed from the solution before titration, by boiling till precipitated and filtering.

Since the total volume of the fluid used in this experiment was 1650cc., and this volume contained 80cc. N/10, H_2SO_4 , every 100cc. contain 0.024 gram of sulphuric acid, which is practically the quantity recommended by Lawrow. This experiment shows, therefore, that more sulphuric acid is necessary to precipitate globin from ox-blood than from the hæmoglobin of the horse.

It should be mentioned that these experiments, in which an exact quantity of sulphuric acid was used, were carried out after I had previously ascertained, by analysis of the precipitated globin, that the substance contained a considerable amount of chemically combined sulphuric acid, a conclusion which was arrived at independent of Lawrow's work. At that time, the globin was precipitated in the presence of a larger amount of sulphuric acid, 2cc. per litre, for 50cc. of 50% solution of ox-blood.

EXPERIMENT 2. — *On the weight of globin obtainable from ox-blood.*

Method.—The Lawrow method was modified by the use of a larger excess of sulphuric acid. The details of the operation were also different, and are described below.

Defibrinated ox-blood was used in all the experiments, the oxygen capacity of the blood being previously determined. In order to avoid any loss, the operation of centrifuging the corpuscles and washing away the serum was carried out on a number of small lots of blood which were subsequently united. Twelve lots of defibrinated blood, 50cc., were carefully measured, and

washed in the centrifuge with normal saline 0.9% NaCl, until the washings gave no precipitate with potassium ferrocyanide and acetic acid, or with nitric acid. The corpuscles were then dissolved in distilled water, the contents of each tube being made up to 100, and the solutions filtered. Of these filtrates, 50cc., therefore, represent the hæmoglobin from 25cc. of defibrinated ox-blood. Four lots of 100cc. of the filtrate were taken and precipitated as below. These experiments were carried out during the progress of other work, and, owing to the fact that about two months were taken up in the drying of the globin precipitates, a somewhat unique preparation was obtained. The details are as follows. Of the hæmoglobin solution, 100cc. were poured gradually into 1600cc. of a mixture of alcohol, ether, and water in the proportions 5: 2: 1, the solution having been previously acidified with 2cc. of sulphuric acid. The flocculent precipitate was allowed to settle. The dark-coloured solution was then removed by suction through cloth stretched over the broad end of a thistle funnel. This method is rapid, and permits of the precipitate being repeatedly washed without incurring any loss, as would happen using filter paper. The washing was continued until the fluid was no longer coloured. The precipitates were then transferred to weighed centrifuge-tubes, and the washing finished in the centrifuge with absolute alcohol, and finally with ether. The precipitates when centrifuged, occupied about 70cc. in volume. The tubes were now placed in the incubator at 37°C., where they remained for six weeks. In order to assist in the drying, vessels containing quick lime were also placed in the warm chamber. At the end of this time, the tubes were transferred to an oven at 50°C., and remained there for two more weeks. The tubes were now of constant weight. The material occupied about 20cc., and was of the consistency of partially baked porcelain. The preparation certainly appeared different from the material previously prepared in the laboratory, and the conclusion seemed justifiable that we were dealing with pure anhydrous globin, or, as it was subsequently shown to be, globin sulphate. It is a difficult matter to remove all the water from the globin prepared in the ordinary way and dried quickly at

100°C., and it is probable that, even when heated to 100°C. for many days, the preparation still contains water in some form of combination. It is interesting to note, therefore, the nature of the precipitate prepared by very gradual dessication lasting about two months, and at a temperature never exceeding 50°C.

The weights obtained were as follows :

Globin sulphate from 50cc. defibrinated ox-blood.

- (a) 8.94 grams.
- (b) 8.84 „
- (c) 8.38 „
- (d) 8.34 „
- (e) 8.48 „

It was found, unfortunately, that Nos. (a) and (b) contained particles of glass, and they were, therefore, not included in calculating the mean value. The last estimation, No. (e), was carried out in two days by drying the globin in an open glass vessel at 110°C.

The percentage of globin sulphate yielded by ox-blood is, therefore,

	Globin sulphate weighed from 100cc. ox-blood.		Calculated to original globin.
No. 1... ..	16.76	15.40
No. 2.....	16.68	15.32
No. 3.....	16.94	15.58
Mean	16.79	Mean	15.43

The figures in the second column are found by deducting the amount of combined sulphuric acid, viz. 1.36 grams determined in the manner to be described later, from the figures of the first column.

There is a good indication, therefore, that the Lawrow method of obtaining globin sulphate gives a product of constant composition.

This is further borne out by the determination of the nitrogen and sulphur in many specimens of the sulphate obtained under various conditions, as regards the concentration of sulphuric acid in the precipitating fluid.

EXPERIMENT 3. *The sulphur in globin and globin sulphate from ox-blood.*

The total sulphur in the globin sulphate was determined by moistening with a strong solution of KOH, evaporating to dry-

ness, and subsequently fusing with an additional quantity of Na_2CO_3 and KNO_3 ; the sulphates were precipitated in hot solution by addition of BaCl_2 .

The precipitates were collected on filter paper, and, after washing and drying, were ignited, and the ash reheated with a few drops of concentrated sulphuric acid.

a) 0.7111 gram globin sulphate gave	0.1610 gram BaSO_4	S 3.11%
(b) 0.7239 gram " " "	0.1747 " "	S 3.29%
* (c) 0.2285 gram " " "	0.0496 " "	S 2.98%
(d) 0.5500 gram " " "	0.1134 " "	S 3.01%
Mean value for sulphur		S 3.05%

It was next of importance to determine how much of this sulphur was present in the globin sulphate as sulphate, and how much as sulphur itself.

An attempt was made to determine this by difference, but the result was not satisfactory, the percentage of organically combined sulphur being found to be too high.

The percentage of sulphur was, therefore, determined in a sample of globin prepared by precipitation in the absence of sulphuric acid. The result gave 0.45 as the mean percentage of sulphur in globin. From this figure, and the data given above for the total sulphur, in globin sulphate, it is possible to calculate the proximate composition of globin sulphate.

EXPERIMENT 4.—*The direct determination of the organically combined sulphur in globin of ox-blood.*

Preparation of the globin :

Alcohol.....	500cc.
Ether.....	200cc.
Water.....	100cc.
Trichloroacetic acid.....	10grams.
Ox-blood 50% solution.....	50cc.

The blood-solution was poured into the acid alcohol-ether mixture, and the solution set aside for some days. No pre-

* I am indebted to Dr. J. M. Petrie, F.I.C., for this sample (c) of globin, which was prepared by him last year. I would express my thanks to him also for many suggestions relating to the Lawrow method.

precipitate appeared; water was now gradually added, and the precipitate, which thus separated, was filtered and washed with alcohol-ether mixture.

25 cc. ox-blood gave 3.96 grams protein—15.84 per cent.

Data of Sulphur Determination.

(1) 0.2700 gram protein gave 0.0094 gram BaSO_4	$\text{S} = 0.47\%$
(2) 0.6460 gram protein gave 0.0200 gram BaSO_4	$\text{S} = 0.43\%$
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Mean $\text{S} = 0.45\%$	

From this value for organically combined sulphur in globin, it is possible, as shewn below, to calculate the distribution of the sulphur in globin sulphate, and also the proportions in which the globin and the sulphuric acid stand related.

In globin sulphate.

Organically combined sulphur.....	0.41%
Sulphur as sulphuric acid.....	2.64%
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Total sulphur	3.05%

Calculation of the composition of globin sulphate.

Assuming 100 parts globin sulphate to contain x parts globin, since, by experiment, globin contains 0.45 per cent. S.

Then the amount of organically combined sulphur in 100 parts of globin sulphate is equal to $0.0045x$.

Again 100 parts of the sulphate contain $(100-x)$ of H_2SO_4 .

Therefore, sulphur present as H_2SO_4 , is equal to $(100-x)\frac{1.6}{49}$ per cent.

But $(100-x)\frac{1.6}{49} + 0.0045x = \text{total sulphur in globin sulphate}$; and, by experiment, the total sulphur was found to be 3.05% .

$$\text{Hence } (100-x)\frac{1.6}{49} + 0.0045x = 3.05$$

$$100-x + 0.0137x = 3.05$$

$$0.9863x = 90.667$$

$$x = 91.92$$

Hence for globin sulphate :

Globin.....	91.92 per cent.
Sulphuric acid.....	8.08 ,,

The determination of the nitrogen in the sulphate of globin from ox-blood, and the calculation of the nitrogen in the original globin.

The nitrogen was determined by Kjeldahl :

(1) 0.2635 gram of sulphate of globin required	...	27.6cc.	N/10 H ₂ SO ₄	N 14.62%
(2) 0.1902 gram	„	20.3cc.	„	N 14.94%
(3) 0.3897 gram	„	41.2cc.	„	N 14.80%
(4) 0.5863 gram	„	62.1cc.	„	N 15.00%
Mean of three determinations				N 14.91%

16.79 grams of globin sulphate are equivalent to 15.66 grams of globin.

Hence the percentage of nitrogen in globin..... N 15.98%

Calculated to ash-free globin..... N 16.03%

The percentage of nitrogen in globin from horse-hæmoglobin, according to Shulz*..... N 16.89%

Determination of the ash.

0.2437 gram of globin sulphate gave 0.0008 gram ash, 0.32%.

The ash found by Shulz in globin from horse-blood was 0.58% 0.84 %.

GENERAL CONCLUSIONS.

- (1) Each hundred cc. of ox-blood yield 16.79 grams of globin sulphate, which is equivalent to 15.43 grams of globin.
- (2) The globin from ox-blood is more basic than that from the hæmoglobin of the horse.
- (3) The sulphate of globin precipitated from solutions containing varying concentrations of sulphuric acid is of constant composition, containing 8.08% sulphuric acid.
- (4) The percentage of nitrogen found in the globin sulphate is 14.9, from which the calculated percentage of nitrogen in the globin from the blood of the ox is 16.03%.
- (5) The globin precipitated by trichloroacetic acid was found to contain 0.45 per cent. sulphur.

In conclusion, I beg to express my thanks to Professor Anderson Stuart, in whose laboratory this work has been done.

* H. S. Zeit. Physiol. Chem. Bd. 24, S.449, 1898.